531 Rec'd PCT/PT 19 JUN 2001

FORM PTO-1390 (REV 11-2000) U S DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. § 371

ATTORNEY'S DOCKET NUMBER: 24615-20146.00

U.S. APPLICATION NO. (If known, see 37 CFR 1.5): 09/869067

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCt/NL99/00782	17/12/99	22/12/98

TITLE OF INVENTION

PROCESS FOR THE PREPARATION OF α-AMINONITRILES WITH ENHANCED OPTICAL PURITY

APPLICANT(S) FOR DO/EO/US

Peter J. QUAEDFLIEG, et al.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.
4. ☐ The US has been elected by the expiration of 19 months from the priority date (PCT Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. ☒ is attached hereto (required only if not communicated by the International Bureau).
   b. ☐ has been communicated by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application under PCT Article 19 (35 U.S.C. 371(c)(2)).
   a. ☐ is attached hereto.
   b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
7. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)).
   a. ☐ are attached hereto (required only if not communicated by the International Bureau).
   b. ☐ have been communicated by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☐ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☒ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☒ A FIRST preliminary amendment.
14. ☐ A SECOND or SUBSEQUENT preliminary amendment.
15. ☐ A substitute specification.
16. ☐ A change of power of attorney and/or address letter.
17. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
18. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
19. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
20. ☐ Other items or information: *; return receipt postcard.

CERTIFICATE OF MAILING BY "EXPRESS MAIL"

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K. S. Huff

d-44911

APPLICATION NO. (if known, see 37 CFR 1.5)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
09/869067	PCT/NL99/00782	24615-20146.00

1. ☒ The following fees are submitted:

BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(5)):

Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO.....$1,000.00

International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO.....$860.00

International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO.....$710.00

International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provision of PCT Article 33(1)-(4) $690.00

International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) $100.00

	CALCULATIONS PTO USE ONLY	
ENTER APPROPRIATE BASIC FEE AMOUNT =	$860.00	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$*	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	$*	
Total claims	21 - 20 =	1	x $18.00	$18.00	
Independent claims	1 - 3 =	*	x $80.00	$*	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ $270.00	$*	
TOTAL OF ABOVE CALCULATIONS =				$878.00	
☐ Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by ½.				$*	
SUBTOTAL =				$878.00	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f)). +				$*	
TOTAL NATIONAL FEE =				$878.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00 per property** +				$40.00	
TOTAL FEES ENCLOSED =				$918.00	
				Amount to be refunded:	**$***
				charged:	**$***

a. ☒ A check in the amount of $918.00 to cover the above fees is enclosed.

b. ☐ Please charge my **Deposit Account No. 03-1952** in the amount of $* to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees that may be required, or credit any overpayment to **Deposit Account No. 03-1952**. A duplicate copy of this sheet is enclosed.

d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

Kate H. Murashige
Morrison & Foerster LLP
3811 Valley Centre Drive
Suite 500
San Diego, California 92130-2332

Reg No. 39,183

SIGNATURE

for Kate H. Murashige
Registration No. (29,959)

d-44911

PATENT

Docket No. 246152014600

CERTIFICATE OF MAILING BY "EXPRESS MAIL"

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K.S. Huff

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re: U.S. National Phase application of:

Peter J. Quaedflieg, *et al.*

Serial No.: Not yet assigned
Based on PCT Intl'l App: PCT/NL99/00782

Int' Filing Date: December 17, 1999

For: PROCESS FOR THE PREPARATION OF α-AMINONITRILES WITH ENHANCED OPTICAL PURITY

Examiner: Not yet assigned

Group Art Unit: Not yet assigned

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

This is a preliminary amendment prior to examination, please amend the claims as follows:

Enclosed is the following Exhibit A:

Exhibit A: Marked-up Version of Amendments to the Claims.

AMENDMENT

Please replace presently pending claims 1-12 with the following claims 1-12:

1. (Amended) A process for preparing an α-aminonitrile with enhanced optical purity which process comprises contacting a mixture of the enantiomers of a chiral N-formyl α-aminonitrile with an acylase, whereby one of the enantiomers of the N-formyl-α-aminonitrile is selectively deformylated into the unprotected corresponding α-aminonitrile.

2. (Amended) A process for preparing an α-aminonitrile with enhanced optical purity which process comprises contacting a mixture of the enantiomers of a chiral (unprotected) α-aminonitrile with an acylase and a formylating agent whereby one of the enantiomers is selectively converted to the corresponding N-formyl α-aminonitrile.

3. (Amended) The process of claim 2 wherein the formylating agent is formic acid, a formic acid amide or a formic acid ester.

4. (Amended) The process of claim 1, wherein the acylase is a peptide deformylase having a bivalent metal ion cofactor from group 5-11 of the periodic system.

5. (Amended) The process of claim 1, wherein the peptide deformylase is chosen from the class EC 3.5.2.27 or EC 3.5.1.31.

6. (Amended) The process of claim 1, wherein the peptide deformylase contains the sequences (I) HEXXH, (ii) EGCLS and (iii) GXGXAAXQ.

7. (Amended) The process of claim 4, wherein the peptide deformylase is from *Escherichia coli.*

8. (Amended) The process of claim 4, wherein the bivalent metal is Fe, Ni, Mn or Co.

9. (Amended) The process of claim 8, wherein the bivalent metal is Ni.

10. (Amended) The process of claim 1, which further comprises adding a stabilisation agent.

11. (Amended) The process of claim 10 wherein the stabilisation agent is catalase.

12. (Amended) The process of claim 10 wherein the bivalent metal is Fe.

Please add the following new claims:

13. (New) The process of claim 2, wherein the acylase is a peptide deformylase having a bivalent metal ion cofactor from group 5-11 of the periodic system.

14. (New) The process of claim 2, wherein the peptide deformylase is chosen from the class EC 3.5.2.27 or EC 3.5.1.31.

15. (New) The process of claim 2, wherein the peptide deformylase contains the sequences (I) HEXXH, (ii) EGCLS and (iii) GXGXAAXQ.

16. (New) The process of claim 13, wherein the peptide deformylase is from *Escherichia coli.*

17. (New) The process of claim 13, wherein the bivalent metal is Fe, Ni, Mn or Co.

18. (New) The process of claim 17, wherein the bivalent metal is Ni.

19. (New) The process of claim 2, which further comprises adding a stabilisation agent.

20. (New) The process of claim 19 wherein the stabilisation agent is catalase.

21. (New) The process of claim 19 wherein the bivalent metal is Fe.

REMARKS

The claims have been amended to eliminate multiple claim dependencies and to conform to U.S. practice. The changes to the claims are editorial and do not constitute new matter. Entry of the amendment is respectfully requested.

In the unlikely event that the transmittal letter is separated from this document and the Patent Office determines that an extension and/or other relief is required, applicants petition for any required relief including extensions of time and authorize the Assistant Commissioner to charge the cost of such petitions and/or other fees due in connection with the filing of this document to **Deposit Account No. 03-1952** referencing docket No. 246152014600. However, the Assistant Commissioner is not authorized to charge the cost of the issue fee to the Deposit Account.

Respectfully submitted,

Dated: June 19, 2001

By: for Kate H. Murashige Reg No 39,183
Registration No. 29,959

Morrison & Foerster LLP
3811 Valley Centre Drive,
Suite 500
San Diego, California 92130-2332
Telephone: (858) 720-5112
Facsimile: (858) 720-5125

EXHIBIT A. - VERSION WITH MARKINGS TO SHOW CHANGES MADE

1. (Amended) [Process] A process for [the preparation of] preparing an α-aminonitrile with enhanced optical purity [wherein] which process comprises contacting a mixture of the enantiomers of a chiral N-formyl α-aminonitrile [is brought into contact] with an acylase, whereby one of the enantiomers of the [N-formylaminonitrile] N-formyl-α-aminonitrile is selectively deformylated into the unprotected corresponding α-aminonitrile.

2. (Amended) [Process] A process for [the preparation of] preparing an α-aminonitrile with enhanced optical purity [wherein] which process comprises contacting a mixture of the enantiomers of a chiral (unprotected) α-aminonitrile [is subjected to a formylation reaction in the presence of] with an acylase and a formylating agent whereby one of the enantiomers is selectively converted [in] to the corresponding N-formyl α-aminonitrile.

3. (Amended) [Process] The process [according to] of claim 2 wherein the formylating agent is formic acid, a formic acid amide or a formic acid ester [is used as a formylating agent].

4. (Amended) [Process] The process [according to any] of [claims 1-3] claim 1, wherein the acylase is a peptide deformylase [with] having a bivalent metal ion cofactor [wherein the metal is chosen] from group 5-11 of the periodic system[, is used as acylase].

5. (Amended) [Process] The process [according to any] of [claims 1-4] claim 1, wherein the peptide deformylase is chosen from the class EC 3.5.2.27 or EC 3.5.1.31.

6. (Amended) [Process] The process [according to any] of [claims 1-5] claim 1, wherein the peptide deformylase contains the sequences [of] (I) HEXXH, (ii) EGCLS and (iii) GXGXAAXQ.

7. (Amended) [Process] The process [according to any] of [claims 4-6] claim 4, wherein the peptide deformylase is from *Escherichia coli.*

8. (Amended) [Process] The process [according to any] of [claims 4-7] claim 4, wherein the bivalent metal is [chosen from the group of] Fe, Ni, Mn [and] or Co.

9. (Amended) [Process] The process [according to] of claim 8, wherein the bivalent metal is Ni.

10. (Amended) [Process] The process [according to any] of [claims 1-8] claim 1, [wherein in addition] which further comprises adding a stabilisation agent[is added].

11. (Amended) [Process] The process [according to] of claim 10 wherein the stabilisation agent is catalase.

12. (Amended) [Process] The process [according to] of claim 10 [or 11] wherein the bivalent metal is Fe.

C L A I M S

1. Process for the preparation of an α-aminonitrile with enhanced optical purity wherein a mixture of the enantiomers of a chiral N-formyl α-aminonitrile is brought into contact with an acylase, whereby one of the enantiomers of the N-formylaminonitrile is selectively deformylated into the unprotected corresponding α-aminonitrile.

2. Process for the preparation of an α-aminonitrile with enhanced optical purity wherein a mixture of the enantiomers of a chiral (unprotected) α-aminonitrile is subjected to a formylation reaction in the presence of an acylase and a formylating agent whereby one of the enantiomers is selectively converted in N-formyl α-aminonitrile.

3. Process according to claim 2 wherein formic acid, a formic acid amide or a formic acid ester is used as a formylating agent.

4. Process according to any of claims 1-3, wherein a peptide deformylase with a bivalent metal ion wherein the metal is chosen from group 5-11 of the periodic system, is used as acylase.

5. Process according to any of claims 1-4, wherein the peptide deformylase is chosen from the class EC 3.5.2.27 or EC 3.5.1.31.

6. Process according to any of claims 1-5, wherein the peptide deformylase contains the sequences of (I) HEXXH, (ii) EGCLS and (iii) GXGXAAXQ.

7. Process according to any of claims 4-6, wherein

the peptide deformylase is from *Escherichia coli*.

8. Process according to any of claims 4-7, wherein the bivalent metal is chosen from the group of Fe, Ni, Mn and Co.

9. Process according to claim 8, wherein the bivalent metal is Ni.

10. Process according to any of claims 1-8, wherein in addition a stabilisation agent is added.

11. Process according to claim 10 wherein the stabilisation agent is catalase.

12. Process according to claim 10 or 11 wherein the bivalent metal is Fe.

A B S T R A C T

Process for the preparation of an α-aminonitrile with enhanced optical purity wherein a mixture of the enantiomers of a chiral N-formyl α-aminonitrile is brought into contact with an acylase, whereby one of the enantiomers of the N-formylaminonitrile is selectively deformylated into the unprotected corresponding α-aminonitrile, and a process for the preparation of an α-aminonitrile with enhanced optical purity wherein a mixture of the enantiomers of a chiral (unprotected) α-aminonitrile is subjected to a formylation reaction in the presence of an acylase and a formylating agent whereby one of the enantiomers is selectively converted in N-formyl α-aminonitrile. Preferably a peptide deformylase with a bivalent metal ion wherein the metal is chosen from group 5-11 of the periodic system, is used as acylase, for instance a peptide deformylase chosen from the class EC 3.5.2.27 or EC 3.5.1.31. Such peptide deformylases often contain the sequences of (I) HEXXH, (ii) EGCLS and (iii) GXGXAAXQ.

The bivalent metal is preferably chosen from the group of Fe, Ni, Mn and Co, in particular Ni or Fe.

PROCESS FOR THE PREPARATION OF OPTICALLY ACTIVE ALPHA-AMINONITRILES

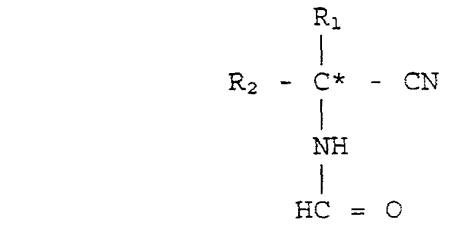
The invention relates to a process for the preparation of an α-aminonitrile with enhanced optical purity wherein a mixture of the enantiomers of the N-formyl-α-aminonitrile is brought into contact with an acylase, whereby one of the enantiomers of the N-formyl α-aminonitrile is selectively deformylated into the unprotected corresponding α-aminonitrile.

There are no processes known in the art wherein a mixture of the enantiomers of an α-aminonitrile is enzymatically, enantioselectively formylated, or wherein a mixture of the enantiomers of an N-formyl-α-aminonitrile is deformylated.

Applicant now has found that it is possible to remove the N-protecting formyl group enantioselectively from one of the enantiomers of a mixture of the enantiomers of N-formyl-α-aminonitriles. In such enantioselective processes moreover very high E-values can be obtained.

α-Aminonitriles to be used as a substrate in the process of the invention are for instance aliphatic and aromatic α-aminonitriles, for example the α-aminonitriles derived from phenylglycine, phenylalanine, *m*-methoxy-phenylalanine, valine and α-methyl-phenylglycine. In the framework of this invention an α-aminonitrile is understood to be an α-aminoacid wherein the carboxy group is replaced by a cyano group.

N-formyl-α-aminonitriles to be used as a substrate are for instance nitriles of formula 1



(1)

wherein:

R_1 represent an, optionally substituted, alkyl or aryl group

R_2 represents H, an, optionally substituted, alkyl or aryl group.

The alkyl groups in R_1 and R_2 may be cyclic or linear or branched chains. The alkyl and aryl groups may be substituted. Suitable substituents are for instance, hydroxy, alkyl, alkoxy, e.g. methoxy, mercapto, alkylmercapto, amino, guanyl, carboxamide, halogen, e.g. chloro, aryl e.g., phenyl and hydroxy phenyl, imidazolyl or indolyl.

In another embodiment of the present invention a mixture of the enantiomers of a (non-protected) α-aminonitrile is subjected to a formylation in the presence of an acylase and a formylating agent, whereby one of the enantiomers is selectively converted into the corresponding N-formyl α-aminonitrile.

Suitable formylating agents are for instance formic acid in case a thermodynamically controlled formylation can be performed, or formic acid esters or amides when the formylation is kinetically controlled. In a thermodynamically controlled formylation the equilibrium is shifted towards the side of the formyl derivative, preferably by precipitation

of the formyl derivative.

Moreover it appeared that, starting from α-aminonitriles, the non-formylated α-aminonitriles relatively rapidly racemise at pH values of higher than 5. In such case the optically active N-formyl aminonitrile can be obtained with an enantiomeric excess of more than 90%, in particular more than 95% and with a yield of more than 90%, calculated with respect to the total amount of (racemic) α-aminonitrile starting product.

Suitable acylases that can be used in the process of the present invention are for instance Penicilline acylases for instance Pen-G or Pen-V acylases, metalloproteases, esterases, deacetylases. Particularly useful enzymes are peptide deformylases.

Peptide deformylases (PDF's) are in general enzymes having formyl methionine peptide deformylase activity. The peptide deformylases to be used according to the invention have a more than 10 times, preferably more than 100 times, in particular more than 1000 times, higher activity towards the N-formyl protected α-aminonitriles compared to the corresponding N-acetyl protected α-amino nitriles. Activity here is defined as the catalytic efficiency (also called: specificity constant) K_{cat}/K_m expressed in M^{-1} sec^{-1}; wherein K_m (expressed in mM) represents the Michaelis constant (this is the substrate concentration at which the reaction rate is 50% of the maximum reaction rate observed) and K_{cat} (expressed in min^{-1}) represents the turnover number. It should be noticed that in the literature also other names are being used instead of the name peptide deformylases; in particular the following names may be mentioned here: formylmethionine

deformylase, N-formylmethionylaminoacyl-tRNA deformylase, N-formyl-L-methionine amidohydrolase, N-formylmethionyl-aminoacyl-tRNA amidohydrolase.

Suitable peptide deformylases to be used in the process according to the invention are peptide deformylases classified as EC 3.5.1.27. Preferably, the enzyme is an enzyme having the activity as described for EC 3.5.1.27 because excellent results are being achieved in the deformylation with such enzymes. It should be noticed that until recently it was believed that the enzyme coded as EC 3.5.1.31 is catalyzing a different reaction. In the meantime, however, it has been shown that the enzymes known as EC 3.5.1.27 and EC 3.5.1.31 are coded for by exactly the same gene and have the same activity. Therefore, as used herein, the term EC 3.5.1.27 is encompassing not only EC 3.5.1.31, but likewise all other enzymes having the same activity as described for EC 3.5.1.27.

Although the family of PDF's is composed of proteins with a relatively low level of sequence identity, the 3D structures of the members of this family appear closely related one to each other with, in particular, the building of a common fold around the bivalent metal ion and three signature sequences. As is described (for PDF's indicated as PDF) by Wagner et al., J. Biol. Chem., 273, 11413-6 (1998), for many of these enzymes characteristically three short amino acid stretches are present as strictly conserved motifs, namely in that the enzymes contain the sequences (i) HEXXH, (ii) EGCLS and (iii) GXGXAAXQ. In these sequences X represents any natural amino acid, and standard one letter codes for amino acids are used: A = alanine, C = cysteine, E = glutamic acid, G = glycine, H = histidine, L = leucine, S = serine and Q = glutamine.

Peptide deformylases are obtainable for

instance from eubacteria for example *Escherichia coli*, *Bacillus subtilis*, *Clostridium acetobutylicum*, *Clostridium beijerinckii*, *Haemophilus influenzae*, *Thermotoga maritima*, *Thermus aquaticus*, *Thermus thermophilus*, *Calothrix PCC 7601*, *Bacillus stearothermophilus*, or *Lactococcus lactis*. Preferably an enzym obtainable from *Escherichia coli* is used.

Preferably a peptide deformylase is used with a bivalent metal ion whereby the metal is chosen from the groups 5-11 of the periodic system (New IUPAC version; see Handbook of Chemistry and Physics 70th edition, CRC Press, 1989-1990, inner page of cover), as a cofactor. Preferably the metal is chosen from the group of V, Cr, Fe, Ni, Mn, Co, Cu, Pd and Pt, in particular from the group of Fe, Ni, Mn and Co, most preferably Fe or Ni.

Preferably the amount of the bivalent metal ions should be about equivalent to the number of moles of enzyme. Suitably the molar ratio between these bivalent metal ions and the number of PDF molecules is in the range of 0.6 to 1.4, preferably of 0.8 to 1.2, and most preferred the amount of bivalent metal ions is equimolar to the enzyme.

Exchange of the bivalent metal ions in the PDF's in order to obtain PDF enzymes with a co-factor as necessary for the present invention can be done by the various methods as described in Groche *et al.*, Biochem. Biophys. Res. Comm., **246**, 342-346, (1998). These methods include simple metal displacement by incubation of the native enzyme in an excess of the desired bivalent metal ion, if necessary preceeded by

the preparation of the apoenzyme via treatment of the native enzyme with a metal chelation compound. Furthermore, the desired bivalent metal ion can already be introduced in (at least part of the enzyme molecules) by using a bacterial growth medium with an enhanced ratio of the desired bivalent metal ion over Fe^{2+}.

In addition measures may be taken in order to enhance the stability of the enzyme, for instance the addition of stabilisation agents, for instance catalase, tris-(2-carboxyethyl)phosphine, glucose oxidase, or combinations thereof; or enlarging the concentration of the PDF, for instance to a PDF concentration of at least 0.1 mg of PDF per ml, more preferably of at least 1.0 mg/ml. The upper limit of the concentration of PDF is not critical if practical concentrations are being used. The use of stabilisation measures is especially preferred when an easily oxidisable metal ion, e.g. Fe^{++} is present as a cofactor or an easily oxidisable substrate. If not, for instance in case Ni^{++} is present as a cofactor, the addition of a stabilisation agent appeared to be superfluous, as the enzyme turned out to be very stable even without stabilisation agent.

In addition measures may be taken in order to enhance the stability of the enzyme, for instance the addition of stabilisation agents, for instance catalase, tris-(2-carboxyethyl)phosphine, glucose oxidase, or combinations thereof; or enlarging the concentration of the PDF, for instance to a PDF concentration of at least 0.1 mg of PDF per ml, more preferably of least 1.0 mg/ml. The upper limit of the

concentration of PDF is not critical if practical concentrations are being used. The use of stabilisation measures is especially preferred when an easily oxidisable metal ion, e.g. Fe^{++} is present as a cofactor or an easily oxidisable substrate. If not, for instance in case Ni^{++} is present as a cofactor the addition of a stabilisation agent appeared to be superfluous, as the enzyme turned out to be very stable even without stabilisation agent.

Alternatively, genetically engineered mutants of PDF's may be used which have for instance enhanced activity or enantioselectivity in the (de)formylation reaction. These mutants can be generated by a number of different approaches; for instance, by site-directed mutagenesis, site-specific random mutagenesis, regio-specific random mutagenesis, and completely random mutagenesis; the latter form of mutagenesis is better known as directed evolution. General applicable methods to perform these different protein engineering approaches are well known to the skilled man. If a random approach will be applied, the mutagenesis cycle will need to be followed by selection of resistent and active mutant(s), thereby leading to the identification of suitable mutants. To obtain PDF mutants also a combination of different protein engineering approaches and/or several rounds of random mutagenesis may be used.

The reaction conditions for the enzymatic deformylation or formylation according to the invention are not very critical and may depend on the substrates

used. Any suitable solvent system which is inert towards the PDF may be applied; such solvents include aqueous systems (solutions or slurries) or aqueous systems also containing a water-miscible organic solvent which is inert under the reaction conditions. Aqueous systems, however, are preferred. Also the concentration of the *N*-formyl compound is not critical, and may be for instance in the range of about 0.1 to 1000 mM. It is not necessary that all of the *N*-formyl compound is dissolved; part of it may be present as a slurry. The concentration of the PDF likewise is not very critical, and usually will be at 0.001 to 100 % by weight of the formyl compound, e.g. at about 0.2 mM of PDF. The pH for the reaction preferably is chosen in the range of 4.0 to 11.0, more preferably of 5.0 to 10.0. The optimum pH is determined by the stability of the α-aminonitrile and/or the N-formyl-α-aminonitrile, and/or the stability and/or activity of the enzyme. The person skilled in the art can easily determine the optimum pH-value. The temperature is not very critical, and suitably will be in the range of 10 to 50°C, e.g. at about 37°C, but for thermostable PDF enzymes higher temperatures may be applied.

In those cases wherein the absolute configuration of the (de)formylated enantiomer was determined, it appeared that the S-enantiomer was (de)formylated more rapidly than the R-enantiomer. The optical purity is given by the enantiomeric excess (ee), the enantioselectivity of the enzyme is represented by E, and calculated as k_f/k_s wherein k_f is

defined as the rate constant of (de)formylation of the most rapidly (de)formylated enantiomer and k_s is defined as the rate constant of (de)formylation of the least rapidly (de)formylated enantiomer.

Optionally a salt promoting hydrophobic interactions is added to the reaction mixture, for instance a sulphate, phosphate, sulphite or acetate of ammonium, Rb, K, Na, Cs or Li. Most preferably ammonium sulphate or lithium sulphate is used.

The invention will further be elucidated by the following 3 examples, without being limited thereto.

Abbreviations:

TB medium: 12 g/l of Bacto-Tryptone, Difco; 24 g/l of yeast extract, Difco; 4 g/l of glycerole; 2.3 g/l of KH_2PO_4; 12.5 g/l of K_2HPO_4);

Hepes: *N*-2-hydroxyethylpiperazine-*N*'-2-ethane sulphuric acid;

AEBSF: 2-aminoethyl-p-benzene sulphonyl fluoride;

TCEP: tris-(2-carboxyethyl)-phosphine.

MOPS: 3-(N-morpholino) propane sulphonic acid

MES: 2-(N-morpholino) ethane sulphonic acid

Isolation of PDF(Fe)

For a detailed discussion of the methods used reference is made to Groche et al., BBRC **246**, 342-346 (1998).

PDF(Fe) was isolated from overproducing *E.coli* cells grown at 30°C in 1.6 l TB medium for 14-16 h. About 13 g (wet weight) cell paste were suspended in 26 ml buffer (20 mM Hepes/KOH, 100 mM KF, pH 7.7 supplemented with 10 µg/ml catalase from bovine liver (Boehringer Mannheim) and 1 mM AEBSF, disintegrated by sonication (Branson B12, 20 min) at 0°C and centrifuged at 200.000g for 1 h. The clear supernatant (1.3 g of protein; according to biurete reaction) was mixed with 1.3 ml 10%(w/v) Polymin G-35 (BASF) adjusted to pH 7.7 and centrifuged at 40.000g for 10 min. The supernatant was applied to a 20 ml Met-Lys-Sepharose column that had been equilibrated with 20 mM Hepes/KOH, 100 mM KF, 0.2 mM TCEP, pH 7.7. After washing with 120 ml of 20 mM Hepes/KOH, 100 mM KF, 0.2 mM TCEP, pH 7.7, PDF(Fe) was eluted with 150 ml 20 mM Hepes/KOH, 100 mM KCl, 0.2 mM TCEP, pH 7.7. The protein containing fractions were concentrated by ultrafiltration using an Amicon PM10 membrane (yield: 140 mg protein, 1400 U/mg; determined according to Groche et al). After adjustment of the TCEP concentration to 1 mM and protein concentration to 40 mg/ml, the PDF(Fe) stock solution (40 mg/ml = 2 mM) was stored frozen at -60°C.

After thawing, the PDF(Fe) stock solution could be used directly in the deformylation experiments described below. If however solutions with lower PDF(Fe) concentrations were required for these deformylation experiments, the PDF stock solution was diluted further in 20 mM Hepes/KOH, pH 7.7, 100 mM KCl, 1 mg/ml bovine serum albumin, 10 µg/ml catalase solution.

HPLC-analysis

In all cases HPLC conditions had to be developed in which the two deformylated isomers were separated from each other and from the formylated isomers. To this end two different techniques were applied, i.e. method A and method B, as described below.

From the concentrations of deformylated isomers in the samples after various reaction times, the (de)formylation rate constant (k_f and k_s in $M^{-1}s^{-1}$) could be calculated for both enantiomers, as well as the respective ee values of the deformylated product. The enantioselectivity of the enzyme (E value) was calculated by taking the ratio k_f/k_s and is given, as well as the maximum ee value of the deformylated product observed during the experiments, in the examples below.

Method A (without derivatization)

A Crownpak CR(+) column (4x150 mm) was used. Samples (5 µl) withdrawn from the deformylation mixture were mixed with 95 µl aqueous $HClO_4$ (10 mM) to inactivate PDF(Fe^{2+}). Following a brief centrifugation, 20 µl of the supernatant were applied to the Crownpak CR(+) column. For specific chromatographic conditions and retention times see the examples II and III.

Method B (Precolumn derivatization with o-Phthaldialdehyde (OPA) and N-acetyl-L-cysteine;

(NAC))

Samples (25 µl) withdrawn from the deformylation mixture were mixed with 25 µl aqueous $HClO_4$ (100 mM) to inactivate PDF (Fe^{2+}). Following a brief centrifugation, 40 µl of the supernatant were added to 80 µl 1 M aqueous H_3BO_3/NaOH pH 11, and subsequently 20 µl OPA reagent (consisting of OPA in H_2O/CH_3OH 1:1 v/v with a concentration as indicated in the example) was added, and 10 minutes later 20 µl NAC reagent (consisting of NAC in H_2O/CH_3OH 1:1 v/v with a concentration as indicated in the example) was added. After 5 min derivatization was terminated by addition of 80 µl (500 mM) aqueous H_3PO_4, and 20 µl of the solution were instantaneously applied to a Nucleosil 120-5 C_{18} column (250 x 4 mm). Temperature is ambient and detection is spectrophotometric using a wavelength of 257 nm and/or 340 nm; the used eluent is a mixture of 80 mol% aqueous 0.05 M H_3PO_4 (brought at pH = 7.0 with 1 M NaOH) and 20 vol% CH_3CN.

Example I: Deformylation of *N*-formyl-valine aminonitrile in the presence of Li_2SO_4 at pH = 7.2.

The deformylation reaction of *N*-formyl-valine aminonitrile was performed in a 1.5 ml Eppendorf reaction test tube. The reaction mixture with a total volume of 200 µl contained 100 mM aqueous MOPS/NaOH, 2 M Li_2SO_4 buffer pH 7.2, and 10 mM of *N*-formyl-valine aminonitrile. After thermal equilibration to 37°C the deformylation reaction was started by the addition of

50 µM of PDF. At various reaction times samples of the reaction mixture were withdrawn in which the reaction was stopped by addition of $HClO_4$.

HPLC-analysis was performed according to method B, with [OPA] = 16 mg/ml, and [NAC] = 4 mg/ml, retention times: 8.6 min (L-enantiomeer), 10.2 min (D-enantiomeer).

Results:

E = 47.9

ee_{max} = 95.5

k_s = 0.62 $M^{-1}s^{-1}$

k_f = 29.7 $M^{-1}s^{-1}$

Example II: Deformylation of *N*-formyl-*m*-methoxy-phenylalanine aminonitrile without Li_2SO_4 at pH 7.2

The deformylation reaction of *N*-formyl-*m*-methoxy-phenylalanine aminonitrile was performed as described in example I, with the exception that 100 mM MOPS/NaOH, 250 mM NaCl, 0.1 mg/ml catalase buffer pH 7.2 was used instead of 100 mM MOPS/NaOH, 2 M Li_2SO_4 buffer pH 7.2. Futhermore, 7.2 mM of *N*-formyl-*m*-methoxy-phenylalanine aminonitrile and 2.5 µM of PDF were used.

HPLC-analysis was performed according to method A
Eluent: 90 vol% 10 mM aqueous $HClO_4$/10 vol% CH_3OH
Flow rate: 0.8 ml/min, temperature: 5° C, detection: 210 nm,
retention times:

Deformylated enantiomer(s): 23.8 min.

30.7 min.

N-formyl aminonitrile: 52.0 min.

Results:

E = 685

ee_{max} = 99.0

k_f = 1370 $M^{-1}s^{-1}$

k_s = 2 $M^{-1}s^{-1}$

Example III: Deformylation of *N*-formyl-phenylalanine aminonitrile without Li_2SO_4 addition at pH 6.2.

The deformylation reaction of *N*-formyl-phenylalanine aminonitrile was performed as described in example I, with the exception that 100 mM MES/NaOH buffer pH 6.2 was used instead of 100 mM MOPS/NaOH, 2 M Li_2SO_4 buffer pH 7.2. Furthermore, 7.5 mM of *N*-formyl-phenylalanine aminonitrile and 20 µM of PDF were used.

HPLC-analysis was performed according to method A

Eluent: 90 vol% 10 mM aqueous $HClO_4$/10 vol% CH_3OH

Flow rate: 0.8 ml/min, temperature: 5° C, detection: 210 nm,

retention time:

deformylated aminonitrile: 11.8 min

15.1 min

N-formyl aminonitrile: 28.6 min.

Results:

E = 880

ee_{max} = 98.8

k_f = 880

k_s = 1

C L A I M S

1. Process for the preparation of an α-aminonitrile with enhanced optical purity wherein a mixture of the enantiomers of a chiral N-formyl α-aminonitrile is brought into contact with an acylase, whereby one of the enantiomers of the N-formylaminonitrile is selectively deformylated into the unprotected corresponding α-aminonitrile.

2. Process for the preparation of an α-aminonitrile with enhanced optical purity wherein a mixture of the enantiomers of a chiral (unprotected) α-aminonitrile is subjected to a formylation reaction in the presence of an acylase and a formylating agent whereby one of the enantiomers is selectively converted in N-formyl α-aminonitrile.

3. Process according to claim 2 wherein formic acid, a formic acid amide or a formic acid ester is used as a formylating agent.

4. Process according to any of claims 1-3, wherein a peptide deformylase with a bivalent metal ion wherein the metal is chosen from group 5-11 of the periodic system, is used as acylase.

5. Process according to any of claims 1-4, wherein the peptide deformylase is chosen from the class EC 3.5.2.27 or EC 3.5.1.31.

6. Process according to any of claims 1-5, wherein the peptide deformylase contains the sequences of (I) HEXXH, (ii) EGCLS and (iii) GXGXAAXQ.

7. Process according to any of claims 4-6, wherein

the peptide deformylase is from *Escherichia coli*.

8. Process according to any of claims 4-7, wherein the bivalent metal is chosen from the group of Fe, Ni, Mn and Co.

9. Process according to claim 8, wherein the bivalent metal is Ni.

10. Process according to any of claims 1-8, wherein in addition a stabilisation agent is added.

11. Process according to claim 10 wherein the stabilisation agent is catalase.

12. Process according to claim 10 or 11 wherein the bivalent metal is Fe.

9872US/CNT1/

DECLARATION FOR *UTILITY/DESIGN] PATENT APPLICATION
AS A BELOW-NAMED INVENTOR, (I~) HEREBY DECLARE THAT:

(My) residence, post office address, and citizenship are as stated below next to (my) name(s~).

(I~) believe (i~) (am) the original, first and *[sole/joint] inventor(s~) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

PROCESS FOR THE PREPARATION OF α-AMINONITRILES WITH ENHANCED OPTICAL PURITY .

the specification of which is attached hereto unless the following box is checked:

☒ was filed on 17 December 1999 as PCT International Application No. PCT/NL99/00782.

(I~) hereby state that I have reviewed and understand the contents of the above-mentioned specification, including the claims, as amended by any amendment referred to above.

(I~) acknowledge the duty to disclose information which is material to the patentability as defined in 37 C.F.R. § 1.56.

(I~) hereby claim foreign priority benefits under 35 U.S.C. § 119(a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate, or § 365(a) of any PCT International application which designated at least one country other than the United States listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or PCT International application having a filing date before that of the application on which priority is claimed:

Application No.	Country	Date of Filing (day/month/year)	Priority Claimed?
98204370.5	Europe	22 December 1998	☒Yes ☐No

(I~) hereby claim benefit under 35 U.S.C. § 119(e) of any United States provisional application(s) listed below:/

Application Serial No.	Filing Date
*	

(I~) hereby claim the benefit under 35 U.S.C. § 120 of any United States application(s), or § 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. § 112, (i~) acknowledge the duty to disclose information which is material to patentability as defined in 37 C.F.R. § 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application.

Application Serial No.	Filing Date	Status
PCT/NL99/00782	17 December 1999	☐Patented ☒Pending ☐Abandoned

I hereby appoint the following attorneys and agents to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

Randolph Ted Apple (Reg No. 36,429)
Sanjay S. Bagade (Reg No. 42,280)
Erwin J. Basinski (Reg No. 34,773)
Richard R. Batt (Reg No. 43,485)
Vincent J. Belusko (Reg No. 30,820)
Kimberly A. Bolin (Reg No. 44,546)
Tyler S. Brown (Reg No. 36,465)
A. Randall Camacho (Reg No. 46,595)
Robert K. Cerpa (Reg No. 39,933)
Alex Chartove (Reg No. 31,942)
Thomas E. Ciotti (Reg No. 21,013)
Matthew M. D'Amore (Reg No. 42,457)
Peter Davis (Reg No. 36,119)
Carolyn A. Favorito (Reg No. 39,183)
Hector Gallegos (Reg No. 40,614)
Debra J. Glaister (Reg No. 33,888)
Johney U. Han (Reg No. 45,565)
Alan S. Hodes (Reg No. 38,185)
Peter Hsieh (Reg No. 44,780)
Madeline I. Johnston (Reg No. 36,174)
Parisa Jorjani (Reg No. 46,813)
Richard C. Kim (Reg No. 40,046)
Kawai Lau (Reg No. 44,461)
Michael J. Mauriel (Reg No. 44,226)
Philip A. Morin (Reg No. P-45,926)
Martin M. Noonen (Reg No. 44,264)
Paul J. Riley (Reg No. 38,596)
Terri Shieh-Newton (Reg No. 47,081)
Kevin R. Spivak (Reg No. 43,148)
Michael R. Ward (Reg No. 38,651)
Todd W. Wight (Reg No. 45,218)
David T. Yang (Reg No. 44,415)
George C. Yu (Reg No. 44,418)
Laurie A. Axford (Reg No. 35,053)
Joseph Barrera (Reg No. 44,522)
Shantanu Basu (Reg No. 43,318)
Frank P. Becking (Reg No. 42,309)
Jonathan Bockman (Reg No. 45,640)
Barry E. Bretschneider (Reg No. 28,055)
Nicholas Buffinger (Reg No. 39,124)
Mark R. Carter (Reg No. 39,131)
Peng Chen (Reg No. 43,543)
Thomas Chuang (Reg No. 44,616)
Cara M. Coburn (Reg No. 46,631)
Raj S. Davé (Reg No. 42,465)
Stephen C. Durant (Reg No. 31,506)
David L. Fehrman (Reg No. 28,600)
Thomas George (Reg No. 45,740)
Kenneth R. Glick (Reg No. 28,612)
Douglas G. Hodder (Reg No. 41,840)
Charles D. Holland (Reg No. 35,196)
Wayne Jaeschke, Jr. (Reg No. 38,503)
Richard D. Jordan (Reg No. 33,519)
Ararat Kapouytian (Reg No. 40,044)
Cameron A. King (Reg No. 41,897)
Rimas T. Lukas (Reg No. 46,451)
Gladys H. Monroy (Reg No. 32,430)
Kate H. Murashige (Reg No. 29,959)
Catherine M. Polizzi (Reg No. 40,130)
Debra A. Shetka (Reg No. 33,309)
Rebecca Shortle (Reg No. 47,083)
Stanley H. Thompson (Reg No. 45,160)
E. Thomas Wheelock (Reg No. 28,825)
Frank Wu (Reg No. 41,386)
Peter J. Yim (Reg No. 44,417)
Karen R. Zachow (Reg No. 46,332)

and:

Please direct all communications to:

Morrison & Foerster LLP
3811 Valley Centre Drive
Suite 500
San Diego, California 92130-2332

Please direct all telephone calls to Morrison & Foerster at (858) 720-5100.

(I~) hereby declare that all statements made herein of (my) own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under § 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date

Name: QUAEDFLIEG, Peter, Jan, Leonard, Mario
Residence: The Netherlands
Citizenship: Dutch
Post Office Address: Kochstraat 6, 6164 HB Geleen NLX

May 8th, 2001
Date

Name: SONKE, Theodorus
Residence: The Netherlands
Citizenship: Dutch
Post Office Address: Caeciliastraat 15, 6143 BK Guttecoven NLX

May 25th, 2001
Date

Name: WAGNER, Adolf, Fritz, Volker
Residence: Germany
Citizenship: German
Post Office Address: Vischerstrasse 25, 71638 Ludwigsburg DEX

May 9th, 2001
Date

Name: BROXTERMAN, Quirinus, Bernardus
Residence: The Netherlands
Citizenship: Dutch
Post Office Address: Gelrestraat 11, 6151 JA Sittard NLX

May 15th 2001
Date

Name: BOESTEN, Wilhelmus, Hubertus, Joseph
Residence: The Netherlands
Citizenship: Dutch
Post Office Address: Brountslaan 9, 6132 BJ Sittard